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Activity of Soil Insecticides on Eggs of *Diabrotica undecimpunctata howardi:* Effects on Embryological Development and Influence of Egg Age

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Abstract: The activity of tefluthrin, carbofuran, terbufos and dieldrin in soil against eggs of different ages of the southern corn rootworm, *Diabrotica undecimpunctata howardi* (Barber) (Coleoptera: Chrysomelidae) was determined. Embryological development following treatment with these insecticides was also investigated to determine the stage of developmental arrest. Generally, younger eggs were found to be more susceptible, dieldrin being the least and tefluthrin the most potent ovicide of combined egg age mortalities. Terbufos and carbofuran were relatively inactive against older eggs compared with tefluthrin. Terbufos stopped embryonic development at the time when acetylcholine esterase activity has been shown to commence. Carbofuran, dieldrin and tefluthrin slowed but did not arrest embryonic development, although the two former compounds prevented eclosion to a greater degree than tefluthrin, suggesting death during absorption of serosal fluids at eclosion. Neonate emergence following treatment of eggs with tefluthrin resulted in death within a few hours. The results are discussed in relation to insecticidal action and permeability changes of egg membrane structure with age.

Key words: tefluthrin, carbofuran, terbufos, dieldrin, ovicides, southern corn rootworm

1 INTRODUCTION

The vast majority of insecticides are targeted at the most destructive stage of an insect pest: the larval or nymphal stage, or the imago. These stages are also usually the most accessible for treatment, being generally mobile and visible. Eggs, on the other hand, are immobile and discreet and specific ovicidal treatments may therefore present a problem.

Representatives from various classes of insecticides have been used as ovicides against insect pests. Petroleum oils and dinitrophenols were among the first used and were eventually replaced by the cyclodiene, endrin and DDT.[1] Subsequently, the less persistent organophosphates and carbamates replaced organochlorines, giving added protection against a wider range of insects, some of which were additionally showing resistance to the organochlorines.[2]

Studies on organophosphate ovicidal activity on above-ground pests include those against eggs of *Musca domestica* (L.),[3] *Oncopeltus fasciatus* (Dallas),[4] *Sanninoidea exitiosa* (Say),[5] *Pieris brassicae* (L.)[6] and *Dysdercus koenigii* (F.),[7] while carbamate activity has been studied against eggs of *Acheta domesticus* (L.),[8] *Hylemya brassicae* (Bouché)[9] and *Heliothis virescens* (F.), for example.[10] In one of the very few 'ovicidal' studies on

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soil pests, carbofuran and terbufos were tested against *Diabrotica* spp.[11] although larval, rather than egg, mortality was actually assessed.

The advent of pyrethroids in pest-control programmes yielded ovicidal data for deltamethrin against *Plutella xylostella* (L.)[12] and for various pyrethroids against *D. koenigii*[13] and *Pygaera fulgurita* (Walker).[14] To date, there are no published studies on the ovicidal activity of the novel pyrethroid, tefluthrin. This insecticide represents the first soil-active pyrethroid in use in the field against soil pests and owes its activity in the soil to a higher vapour pressure than other pyrethroids.[15]

While the nervous system is the target of most insecticides, the mode of action of ovicides is complicated by a number of factors:

Firstly, the egg shell or chorion is a barrier to insecticide penetration[16] and beneath this are embryonic membranes that continually change during embryonic development and restrict the movement of compounds;[4,16,17] some of which are absorbed, degraded or eaten on eclosion by the emerging larva.

Secondly, enzymes that are the primary target sites in larvae and adults are not necessarily present in the same form or relative concentration in the eggs. For example, organophosphates exert their toxic effects largely by inhibition of acetylcholinesterase (AChE).[18] In most cases, insect eggs produce AChE late in their development, correlating with increased susceptibility to toxicants,[13,19,20] implying increased susceptibility of older eggs to AChE inhibition. Although this is often the case, it can be an over-simplification.[1] Embryonic development is not usually arrested by ovicides.[1] However, extremely high doses of toxicants that do arrest embryonic development may invoke alternative and unusual modes of action.[2,3]

Prophylactic field treatments for chemical control of *Diabrotica* spp. are generally aimed at larval stages[11,15] and information is lacking on the potential contribution to plant protection of ovicidal effects. In this study, the effects of four insecticides, dieldrin, carbofuran, terbufos and tefluthrin, on development of eggs of the southern corn rootworm, *D. undecimpunctata howardi* Barber are described. Two age classes of eggs were examined in this work to allow for the possible influence of developmental changes (e.g. in membrane structure, neurochemistry) on ovicidal activity.

2 MATERIALS AND METHODS

2.1 Chemicals

Technical grade dieldrin: (1*R*,4*S*,4a*S*,5*R*,6*R*,7*S*,8*S*,8a*R*)-1,2,3,4,10,10-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-6,7-epoxy-1,4 : 5,8-dimethanonaphthalene, 99·0% by weight (Sigma Chemicals); terbufos: *S-tert*-butylthiomethyl *O*,*O*-diethyl phosphorodithioate, 98·6% by weight (American Cyanamid Inc.); tefluthrin: 2,3,5,6-tetrafluoro-4-methylbenzyl (Z)-(1*RS*)-*cis*-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate, 99·0% by weight (ICI (now Zeneca Agrochemicals Ltd)) and carbofuran: 2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate, 99·0% by weight (Riedel-deHaen (Akt.)) were stored at 4°C. Test solutions were prepared in analytical grade acetone and stored at 4°C until use.

2.2 Insects

The laboratory colony of *D. undecimpunctata howardi* was established from a sub-population (ex-May and Baker, UK) originating from a susceptible laboratory-reared stock in the US corn belt. Detailed laboratory rearing methods were derived from a variety of sources and are described elsewhere.[21–23] The adults were kept in clear Perspex cages (100 × 50 × 50 cm) with ventilation holes and maintained at 27(±2)°C and 60–75% RH in a 16 : 8 h light : dark cycle. An artificial diet and fresh Chinese cabbage (*Brassica chinensis* var. *pekinensis*, cv. Tip-Top) leaves were provided as food and water source. The cabbage leaves also acted as an oviposition attraction source and were placed above a metal mesh placed on a plastic pot filled with damp gravel.

2.3 Ovicide bioassays

Eggs laid by two- to eight-week-old beetles were used, as previous work showed adults of this age produced eggs of maximum fertility.[23] Eggs were collected from a plastic oviposition pot filled with damp gravel over a 5–6 h period and kept in damp gravel at 25 (±2)°C for 48 h. This procedure was repeated after 24 h, in the latter case keeping the collected eggs in damp gravel for 24 h, which provided two age groups of eggs (24–30 h and 48–54 h) on the day of test. Each gravel pot was sieved separately through nylon mesh and the eggs gathered on damp filter paper.

All assays were conducted using sieved and air-dried Boughton sandy loam (sand 53%, clay 24%, silt 23%, organic matter 4·47%, pH 7·0) brought to 50% soil moisture content with tap water. Plastic Petri dishes (7 cm diam.) were filled with 50 g of soil. An aliquot of insecticide solution (1 ml) was pipetted evenly over the soil surface using an electronic pipette in titration mode and the soil was then left uncovered for 2–3 h to ensure solvent evaporation. A small well was then made in the centre of the soil and eggs (15–25) added using a fine, damp camel-hair brush. The brush was cleaned in acetone between treatments to prevent cross-con-

tamination. The dishes were covered with a pierced lid and held at 25 (±2)°C in covered trays to keep them in the dark and with water in the base to maintain soil moisture content. Three replicates of at least six doses of each insecticide were used, together with acetone alone and untreated soils as controls. Egg hatch was recorded over a 14-day period; remaining eggs were considered to be dead. Records of empty chorions, dead and live larvae and observed abnormalities were also made. When hatch was evident, germinating maize (*Zea mais* L. hybrid field corn AGIO, W. W. Johnson & Son, Boston, Lincs.) was added to the dishes to attract neonates for counting and to prevent the possibility of egg cannibalism. Dead larvae were removed from the soil. Data were transformed where appropriate for egg control mortality.[24] Mortality data were assessed by Logit transformation using the Lawes Agricultural Trust 'Maximum Likelihood Programme' (MLP) for determination of LC_{50} (concentration required to prevent 50% egg hatch) levels.[25]

In a separate assay, three pre-determined treatment levels of ×15, ×2 and ×0·3 (labelled high, medium and low, respectively) of the estimated LC_{50} values for each insecticide against third-instar larvae[23] were used against old and young eggs. These treatment levels were selected to reflect pesticide concentrations likely to be found in the soil following field application. Egg hatch, larval mortality and partial-eclosion deaths were recorded for each of three replicate soil treatments of 20–25 eggs.

2.4 Embryological development

All four insecticides were tested at the LC_{90} level as determined from the above assays in soil together with controls. At least 10 eggs (0–4 h old) were added to each of 20 treatment sets for each compound. All eggs were removed from each of two replicated sets each day over a 1- to 10-day period. Development at each time point was stopped by fixing the eggs in Bouin's solution for 24 h at 37°C followed by three washes in 50% (by volume) ethanol. The eggs were left in the last ethanol wash for 24 h at 37°C and then transferred to 70% ethanol prior to examination.[26] Staining of the embryo material using the methods of Storch and Krysan,[26] was unsuccessful. However, some success was achieved by using a lacto-phenol solution (Phenol + lactic acid + glycerol + water, 1 + 1 + 2 + 1 by weight).[27] The egg shells and membranes were carefully removed under magnification using fine forceps. On a microscope slide, three or four drops of the lacto-phenol solution were mixed with water (1 + 1) and the eggs placed in the solution and left for 15 min at room temperature for partial digestion of the yolk to proceed. Generally, the older the embryo, the less time was required for this process. Heating the slide reduced the time for digestion but also increased the possibility of loss of embryonic material. Eggs were then returned to 70% ethanol for 15 min, stained using a 0·5 g litre^{-1} acid fuschin solution in 70% ethanol and examined under a stereo microscope (Wild M3 series, Wild Heerbrugg, Luton, Beds.) with transmitted light. Differentiation of embryo and yolk using this procedure was superior to the methods of Storch and Krysan.[26] However, neither method adequately resolved the morphological features of the one- to four-day-old embryos for the determination of developmental stage. Eggs older than four days were scored according to the development stages of Storch and Krysan.[26] Undeveloped eggs scored 0; developed eggs up to day 4 were scored 9 as the assumed stage prior to day 5. Hatched eggs scored 20 and included partially eclosed eggs and this score was used to differentiate between fully developed, hatched larvae and fully developed, unhatched larvae. Scores were compared against time, and pairwise between treatments and time using Kruskal–Wallis one-way ANOVA.

2.5 Egg-transfer studies

A further bioassay was conducted using tefluthrin to determine whether embryonic death was due to (a) ingestion of contaminated sub-chorionic serosal fluids and contaminated egg shells or (b) vapour effects of soil insecticides. Soil samples were treated with the LC_{50} concentration of tefluthrin and young eggs (24–30 h old, 10 eggs in each of three replicates) placed in the soil with a camel-hair brush. After four and eight days of exposure, (*c*.5 and *c*.1 day prior to hatch, respectively) subsamples of these eggs were placed on clean soil and replaced by eggs from untreated soil and both sets allowed to complete the remainder of their development. Tefluthrin-alone and control-alone treatments were also established. Mortality and percentage emergence and partial emergence were noted over a 12-day period.

3 RESULTS

3.1 Ovicidal bioassays

Against older eggs, tefluthrin was significantly ($P < 0{\cdot}05$) more toxic than the other insecticides tested (Table 1). There was a marked difference in LC_{50} levels following treatment of the younger eggs. Carbofuran and terbufos were 16 and 30 times more active, respectively, compared with the older eggs at the LC_{50} level

TABLE 1
Ovicidal Activity of Insecticides against Young (24–30 h) and Old (48–54 h) Eggs of *Diabrotica undecimpunctata howardi* in Treated Sandy Loam Soil at 25°C

	Activity ($\mu g\ g^{-1}$ *soil*)					
	24–30-h eggs			*48–54-h eggs*		
Treatment	LC_{50}[a]	*95% FL*	*Slope* ($\pm SE$)	LC_{50}[a]	*95% FL*	*Slope* ($\pm SE$)
Carbofuran	26·2a	10–72	2·1 (0·17)	412a	108–1590	0·95 (0·13)
Terbufos	12·8a	5–35	2·2 (0·18)	380a	100–1449	0·69 (0·15)
Dieldrin	160·7b	59–439	1·0 (0·15)	364a	96–1387	1·11 (0·14)
Tefluthrin	90·8b	33–248	1·3 (0·16)	23b	6–88	1·35 (0·10)

[a] Column data followed by the same letter are not significantly different ($P > 0{\cdot}05$) based on Fisher's LSD test. *F*-test significant difference between egg age and treatment $F_{1,9} = 17{\cdot}4$ ($P < 0{\cdot}05$).

whilst tefluthrin was four times less active. Tefluthrin and dieldrin were significantly ($P < 0{\cdot}05$) less active than terbufos and carbofuran against younger eggs (Table 1). There was a significant ($P < 0{\cdot}05$) difference between the slopes of terbufos-treated young and old eggs. A marked difference of slope was also evident with carbofuran-treated young and old eggs (Table 1). In contrast, the tefluthrin and dieldrin slope responses were similar for both egg age groups.

In the second experiment, tefluthrin treatments at all dose levels resulted in a high incidence of larval death immediately upon eclosion with neonates unable to emerge successfully (Table 2). This effect did not occur with the other insecticides. The results also showed that at concentrations used to kill larvae, there was also some ovicidal activity, although at $2 \times LC_{50}$ (medium) for all the insecticides there was a total kill of only 23–36%

3.2 Embryological development

The mean embryonic development score following treatment of 0- to 4-h-old eggs with insecticides indicated that no further development or hatch of treated eggs occurred beyond day 8 (Fig. 1). There was a significant difference ($P < 0{\cdot}05$) in development score of eggs

TABLE 2
Form of Mortality of *Diabrotica undecimpunctata howardi* Eggs following Treatment of Young (24–30-h-old) and Old (48–54-h-old) Eggs with Various Levels of Insecticides in a Sandy Loam Soil Bioassay at 25°C

		Mean % mortality of each stage					
		24–30-h eggs			*48–54-h eggs*		
Treatment	$\mu g\ AI\ g^{-1}$ *soil*[a]	*Egg mortality*[b]	*Part emerged*[c]	*Larval death*[d]	*Egg mortality*[b]	*Part emerged*[c]	*Larval death*[d]
Carbofuran	75·0	99	0	0	54	1	18
	10·0	29	0	12	36	0	19
	1·6	18	0	4	20	0	0
Terbufos	35·0	94	0	2	40	0	38
	5·0	33	0	9	23	0	11
	0·7	23	0	2	19	0	9
Dieldrin	250·0	58	6	19	67	0	2
	35·0	28	0	14	35	2	14
	5·0	25	0	12	31	0	9
Tefluthrin	150·0	70	52	29	85	11	14
	20·0	27	10	52	35	17	45
	3·0	30	3	61	34	13	30

[a] Doses = × 15, × 2 and × 0·3 third-instar larval soil LC_{50}.[23]
[b] Eggs unhatched after nine days.
[c] Death of larvae while not fully emerged from chorion.
[d] Death within a few hours.

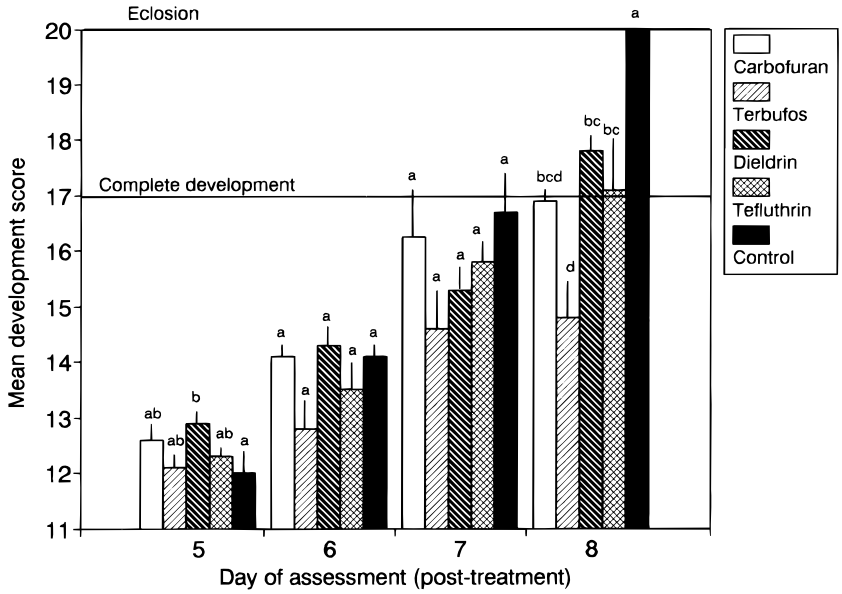

Fig. 1. The development of 0 to 4-h-old eggs of *Diabrotica undecimpunctata howardi* (Barber) treated at LC_{90} levels with a variety of insecticides in soil. Embryo development scored according to the scheme of Storch and Krysan.[26] Scores on each day of assessment with the same letter are not significantly different ($P > 0{\cdot}05$, Kruskal-Wallis one-way ANOVA).

on day 5, between the dieldrin treatments and the control. However, there was no significant difference ($P > 0{\cdot}05$) between treatments and egg development stage on days 6 and 7. Examination of carbofuran, tefluthrin and dieldrin-treated eggs at day 8 showed advanced development but low eclosion. Terbufos-treated eggs stopped development at this time and remained at around stage 15. All insecticide treatments on day 8 showed significant differences ($P < 0{\cdot}05$) in development stages compared with control eggs. Terbufos was, additionally, significantly different ($P < 0{\cdot}05$) compared with tefluthrin and dieldrin-treated eggs. Normal development (full development = score 17) was observed for tefluthrin, carbofuran and dieldrin-treated eggs, although eclosion did not occur. Emergence at day 8 was 20%, 20%, 5%, 0% and 100% in the dieldrin, tefluthrin, terbufos, carbofuran and control treatments, respectively (Fig. 1).

3.3 Egg transfer studies

The results from the transfer studies (Table 3) showed that after four and eight days exposure to tefluthrin there was no significant difference ($P > 0{\cdot}05$) in mortality, both treatments resulting in 65–67% ovicidal activity (slightly higher than the predicted LC_{50} concentration). The work showed that the continual-exposure treatment induced 54% ovicidal action. Transfer of treated-soil eggs to untreated soil resulted in high

TABLE 3
Effect of Tefluthrin treatment (LC_{50}) on 24- to 30-h-old *Diabrotica undecimpunctata howardi* Eggs following Transfer to Treated and Untreated Sandy Loam Soils at 25°C

Treatment	*Mean (%) and range*[a]		
	Egg hatch	*Partial emergence*	*Total egg mortality*
Continual exposure	46 (29–56)	33 (29–38)	54 (44–71)
Control	90 (82–97)	0	10 (3–18)
Four days after exposure			
Control to tefluthrin	32 (29–35)	40 (35–47)	67 (65–71)
Tefluthrin to control	97 (95–100)	0	3(0–5)
Eight days after exposure			
Control to tefluthrin	34 (31–41)	43 (38–50)	65 (59–69)
Tefluthrin to control	91 (88–95)	0	8 (5–12)

[a] $n = 10$ eggs × three replicates.

eclosion similar to controls, suggesting exposure at eclosion was critical.

4 DISCUSSION

The susceptibilities of eggs to the toxicants tested showed a marked difference with age. The change in the slopes of the regression lines (Table 1) suggested an increase in the heterogeneity of the response of older eggs to terbufos and (to a lesser extent) carbofuran treatment, unlike the responses to tefluthrin and dieldrin. The general conclusion from the data is the consistently poor performance of dieldrin; the much greater activity of the AChE inhibitors terbufos and carbofuran against younger compared with older eggs; and the greater activity of tefluthrin against older eggs. The observed action of the organophosphate and carbamate compounds is in agreement with the work of some other authors. For example, young eggs of *Lyctus brunneus* (Stephens) were easier to kill than older eggs[28] and also eggs of *O. fasciatus* were highly susceptible to insecticides in the early stages of embryonic development.[5] Similar conclusions were drawn from *A. domesticus* eggs treated with carbaryl and propoxur, although in this case the solvent used may have contributed to the toxicity observed.[8] However, carbofuran gave a consistent 70% reduction of egg hatch of *Delia radicum* (L.) eggs (3–7 h and 24–48 h old) regardless of age.[29]

It was reported that *M. domestica* eggs treated at an early stage with organophosphates developed normally and died just before emergence.[3] This suggests a latent toxic effect, mortality occurring when the toxicant enters the eggs during membrane degeneration or at a time when AChE has taken on a physiological role. The effects of 10 organophosphates and two carbamates on *O. fasciatus* eggs showed that dichlorvos was the only compound with true ovicidal activity against older eggs.[4] Clearly, susceptibility to organophosphate and carbamate insecticides varies with insect species, toxicant and egg age. The method of exposure is also influential and must be defined in any such tests.

Two of the more likely explanations for the differences in ovicidal activity found in the present work are as follows.

Firstly, changes in membrane form and permeability may explain the differences in activity of terbufos, carbofuran and dieldrin to young and old eggs and are likely to have accounted for the heterogeneous response of older eggs to terbufos and carbofuran treatment. For instance, embryonic membranes of *D. undecimpunctata howardi* eggs were not fully formed until 22 h after egg deposition.[30] Entry of compounds into the target embryo in the young-egg bioassays could therefore have been easier compared with older eggs where the membranes are at a more complete form.[30,31] The development of the AChE system with time would imply an increase rather than a decrease in the activity of terbufos and carbofuran. Consequently, the lack of activity can be assumed to be due to the poor penetration of the chemicals into the eggs.

Secondly, many workers have correlated the activity of organophosphates and carbamates with the appearance of AChE in developing embryos and maturity of the central nervous system in a variety of insect species.[2,19,32,33] However, others have noted kill and reduced hatch at times when AChE is not present. It was proposed, for instance, that when AChE is absent, other enzymes may be attacked.[18,34] Krysan and Guss[18] subsequently found that lipases were sensitive to paraoxon and therefore a possible primary target when AChE was not normally present. They suggested that the reason for *O. fasciatus* egg insensitivity to organophosphates[4] was because eggs of these insects do not rely on fats (and thus lipases) for embryogenesis. In the present work, the observed mortality of young eggs of *D. undecimpunctata howardi* with no appreciable amounts of AChE until day 5 onwards,[18,20] could suggest additional activity on lipases or other esterase enzymes.

The pyrethroid tefluthrin was the most active compound tested in the present study against older eggs. The use of fenvalerate and cypermethrin against younger eggs of *D. koenigii* showed them more resistant to these insecticides.[13] The pyrethroid, deltamethrin, used against *P. fulgurita*[14] and *P. xylostella*[12] also gave similar results to the present work. Interestingly, young and old eggs of *D. koenigii* were found to be less resistant than eggs of intermediate age to the ovicides allethrin and DDT.[13]

The tefluthrin transfer study showed that eggs in tefluthrin-treated-to-untreated soil were unaffected. However, eggs in untreated-to-tefluthrin-treated soil suffered the same fate as eggs kept solely in tefluthrin-treated soil. The time of exposure of eggs to tefluthrin was therefore relatively unimportant and suggests that, at the LC_{50} level at least, larvae are killed as they emerge into a tefluthrin vapour environment. Some workers suggest toxicity of insecticides is influenced by the rate of penetration rather than simple embryonic changes in susceptibility.[4,5] In the present work low-level entry of tefluthrin was possibly being successfully metabolised by the embryo. The activity described for tefluthrin may be similar for dieldrin and carbofuran, as eggs treated with these compounds also had almost fully developed embryos which failed to emerge.

The concentrations used to study the developmental processes (Table 1) were higher at the LC_{90} level than those in the transfer studies which used lower LC_{50} levels. These differences may have influenced the pattern of activity of the compound in these tests, high concentrations resulting in different primary targets (i.e. alternative enzyme targets) or additional modes of action (a

combination of AChE and lipase activity) than normal.[34]

An action of terbufos on young eggs of *D. undecimpunctata howardi via* AChE inhibition was indicated by the development results which showed arrested development of the embryos at a mean score of 14·8. This score represents development to day 5 which is near the time of the appearance of AChE.[20] Carbofuran-, tefluthrin- and dieldrin-treated eggs all reached full development scores of around 17, suggesting that poisoning occurred only when the central nervous system was complete. It was also observed that development rate was slower for the dieldrin, tefluthrin and carbofuran treatments than with controls, implying reduced vigour possibly due to the metabolic costs of detoxification of the insecticides.

In the present work, tefluthrin was found to be active at relatively low concentrations compared with its larvicidal activity in soil bioassays[23] and was highly active against both young and old eggs and neonate larvae at similar concentrations. In contrast, the LC_{50} concentrations of terbufos, carbofuran and dieldrin needed for ovicidal activity of young eggs appeared to be far greater than those required for 3rd-instar larvae in soil. The need for much higher levels of insecticide was also reported for *Epilachna varivestris* (Mulsant) eggs which required ten times the larval LD_{95} for carbofuran for inhibition of hatch.[35] Carbofuran was also found to be a weak ovicide using eggs of the soil-inhabiting pest *H. brassicae*.[9]

Overall, tefluthrin was the most active chemical against older eggs and slightly less active against younger eggs of *D. undecimpunctata howardi.* While the mechanism of action and penetration was difficult to assess, due to the results of the egg-transfer data, the vapour pressure and lipophilic nature of tefluthrin are likely to be very important. Certainly, the relationship between such physicochemical characteristics and field performance of soil insecticides is well known.[36] Terbufos and carbofuran appeared only to be of practical use as ovicides against young eggs. In a related study, prophylactic treatments of rootworm insecticides on maize have suggested that ovicidal activity may play a role in crop protection, although ultimately, when compound degradation increases, larval kill would be the main control measure.[23] The use of tefluthrin may sustain ovicidal activity *via* a high vapour pressure in the soil system and high kill of rootworm neonates. However, this would inevitably depend on the residual activity of the insecticide and the duration of diapause of eggs of different rootworm species.

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